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Extinction coefficient for red-shifted chlorophylls: Chlorophyll *d* and chlorophyll *f* ☆

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ABSTRACT

Both chlorophyll *f* and chlorophyll *d* are red-shifted chlorophylls in oxygenic photosynthetic organisms, which extend photon absorbance into the near infrared region. This expands the range of light that can be used to drive photosynthesis. Quantitative determination of chlorophylls is a crucial step in the investigation of chlorophyll-photosynthetic reactions in the field of photobiology and photochemistry. No methods have yet been worked out for the quantitative determination of chlorophyll *f*. There is also no method available for the precise quantitative determination of chlorophyll *d* although it was discovered in 1943. In order to obtain the extinction coefficients (ε) of chlorophyll *f* and chlorophyll *d*, the concentrations of chlorophylls were determined by Inductive Coupled Plasma Mass Spectrometry according to the fact that each chlorophyll molecule contains one magnesium (Mg) atom. Molar extinction coefficient $\varepsilon_{chl\ f}$ is 71.11×10^3 L mol^{-1} $A_{707\ nm}$ cm^{-1} and $\varepsilon_{chl\ d}$ is 63.68×10^3 L mol^{-1} $A_{697\ nm}$ cm^{-1} in 100% methanol. This article is part of a Special Issue entitled: Photosynthesis Research for Sustainability: from Natural to Artificial.

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1. Introduction

Chlorophylls are essential molecules for light-harvesting and energy transduction in oxygenic photosynthesis, which comprises a porphyrin or chlorin ring and hydrocarbon tail, in most cases phytol [1]. The major functions performed by chlorophylls in photosynthetic reactions are: absorbing light efficiently in the light-harvesting complexes; transferring the excitation energy with high quantum efficiency to the reaction centers; and performing the primary charge separation across the photosynthetic membranes and generating membrane potential that leads to ATP and strong reductants (NADPH) [2]. There are five different forms of chlorophylls. Chl *a*, *b* and *c* were identified in the 19th century [3], Chl *d* was reported in 1943, more than 70 years after the other chlorophylls [4]. The fifth chlorophyll, Chl *f* was reported in 2010, 67 years after the last chlorophyll reported [5]. Those five chlorophyll forms show different absorption properties, *i.e.* they can harvest solar light at different wavelengths (Fig. 1). Chl *a* or its 8-vinyl derivative are found in nearly all oxygenic photosynthetic organisms as the major photopigment. Chls *b* or its 8-vinyl derivative and Chl *c* are accessory photopigments, and are only found in light-harvesting chlorophyll-binding complexes. Chl *d* was reported in 1943 and was considered as an artificial product of pigment extraction processes for nearly 60 years until the discovery of *Acaryochloris marina* in 1996 [4,6]. *A. marina* uses Chl *d*, up to 95–99% of total chlorophylls, as its major photopigment [6]. Chlorophyll *d* can replace all function of Chl *a* in *A. marina*, not only in light-harvesting complexes [7,8], but also in reaction centers [9–11]. The redox potential of the special pairs using Chl *d* in *A. marina* was reported at the same level as in Chl *a*-containing cyanobacteria [12,13]. Chl *f*, proposed chemical structure of [2-formyl]-chlorophyll *a*, was isolated from stromatolites, Western Australia [5].

Over the last half century, a reliable assay for quantitative determination of the chlorophylls, including both total chlorophylls and individual chlorophylls, has become an important requirement for the aim of building a realistic model of complete photosynthetic energy-transduction system through obtaining accurate molecular models of chlorophyll–protein complexes [14]. Simultaneously, quantitative determination of these chlorophylls is also important for plant physiology and biochemistry studies associated with various growth conditions [14,15]. The reason for this is that the change of chlorophylls indicated permits the adaption of the chloroplast to the environment by adjusting the number, proportion and size among the different pigment protein complexes in the thylakoid membrane. The chlorophyll assay also provides a quick and reliable method for

Abbreviations: (AAS), Atomic absorption spectroscopy; Chl, Chlorophyll; α, mass extinction coefficient; ε, molar extinction coefficients; (ICP-MS), Inductively Coupled Plasma Mass Spectrometry; L, liter; RSD, Relative standard deviation

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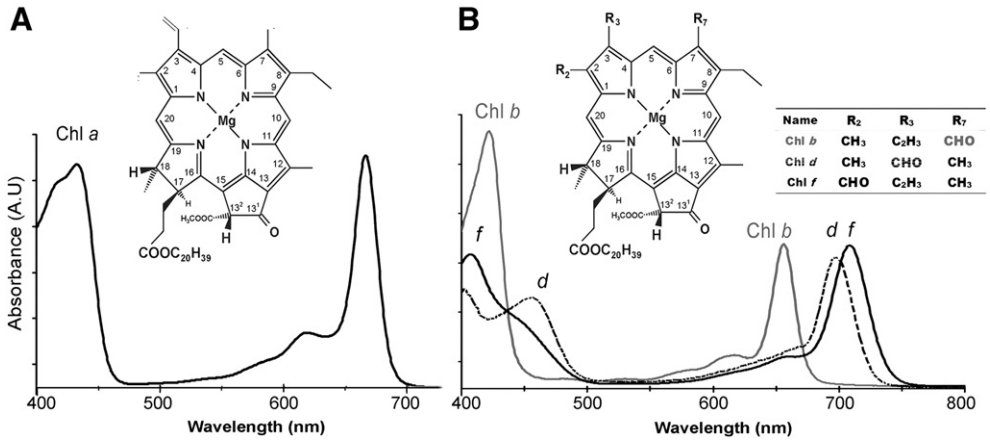


Fig. 1. Chemical structures of chlorophylls and their *in vitro* spectra (in 100% methanol). A, Chl *a* chemical structure and its spectrum in 100% methanol; B, other chlorophyll structure varieties and their spectra recorded in 100% methanol.

estimating the photosynthetic rates. The quantity of chlorophylls, the spectral properties of chlorophylls and their extinction coefficients at certain wavelengths are all essential parameters.

The extinction coefficients, including mass extinction coefficient (α) and molar extinction coefficient (ε), are parameters to define the absorbance intensity at a given wavelength per mass density (g) or molar concentration (mol), respectively [16,17]. It can be determined by using the Beer–Lambert law, $A_\lambda = \varepsilon \cdot c \cdot l$. In this formula, 'A' represents the absorbance of chlorophyll at wavelength ($_\lambda$), which can be obtained using a UV–vis absorbance spectrometer, and '*l*' represents the path length of cuvette (cm) [18]. In order to obtain the molar extinction coefficient (ε), an accurate molar concentration (c) needs to be determined.

In the 1940s, the extinction coefficients of Chl *a* and Chl *b* were first reported by Mackinney [19], and then revised by Arnon in 1949 [20] to construct the equation for simultaneously determining Chl *a* and Chl *b*, which became a fast and convenient spectrophotometric equation used during much of the second half of the last century. These coefficients underestimate chlorophyll concentration assumedly due to the impurity of isolated chlorophylls and the formation of oxidation products during the sample preparation. In 1989, Porra et al. confirmed the coefficients of Smith and Benitez with an error less than $\pm 1\%$ using atomic absorption spectroscopy for determining the concentration of magnesium, and these coefficients were recognized as reliable parameters and have been used by researchers since then [21,22].

Chl *d* was reported with a well-defined spectrum in 100% methanol in 1943 [4]. There are three defined absorption maxima at 696, 456 and 400 nm in methanol that are different from the spectrophotometric properties in ether and petroleum ether [4]. However, the extinction coefficient of Chl *d* was first reported in 1955 using absorption maxima for Chl *d* in diethyl ether ($\lambda = 688$ nm) and magnesium analysis [21]. The currently used molar extinction coefficient of Chl *d* is based on α ($110.4\ L\ g^{-1}\ A_{688nm}\ cm^{-1}$) in diethyl ether [21,23]. Precision of the Chl *d* extinction coefficient has been questioned since the discovery of *A. marina*, a Chl *d*-containing cyanobacterium in 1996 [6]. Chl *f* is a newly discovered chlorophyll, with its spectral absorption maxima at 706 and 406 nm in 100% methanol [5]. There is a need for accurate extinction coefficients for both red-shifted chlorophylls, Chl *d* and Chl *f*.

Acetone is the common choice solvent for chlorophyll assays [15,22,24], since it can give chlorophylls sharp absorption peaks. However, acetone can be a poor extractant for chlorophylls, especially for algae and cyanobacteria [25]. Methanol is the solvent of choice for more efficient extraction from algae and cyanobacteria [25]. Diethyl ether has been commonly used for measuring chlorophylls and many published assigned spectra of chlorophylls were for 100% diethyl ether as solvent [21,22,26]. Diethyl ether is immiscible with water, which is advantageous for extracting chlorophylls from other water-soluble materials. In this study, we investigated the different spectral profiles for these commonly used solvents.

According to the molecular formulae of chlorophylls, one chlorophyll molecule contains one atom of Mg [1], *i.e.* the number of moles of chlorophyll equals the number of moles of Mg in chlorophyll. Atomic spectroscopy techniques exploit the properties of atoms emitting or absorbing element-specific electromagnetic radiation, or having differences in mass [27]. There are three commonly used atomic spectroscopy techniques: atomic absorption spectroscopy (AAS), inductively coupled plasma optical emission spectroscopy (ICP-OES), and inductively coupled plasma mass spectrometry (ICP-MS) [28]. ICP-MS typically has the lowest detection limits of the three techniques and also allows the measurement of isotopic abundance [29,30]. This technique has become the standard method for ultra-trace analysis of metal elements in various materials [31,32]. In this study, the molar concentrations of Mg extracted from purified Chl *f* and Chl *d* were determined using ICP-MS. Here, we have used these values to calibrate and report the extinction coefficients (ε and α) of Chl *f* and Chl *d* in various solvents based on the Beer–Lambert law.

2. Materials and methods

2.1. Extraction of chlorophylls

Three chlorophylls, Chl *a*, Chl *d* and Chl *f* were isolated using pre-chilled 100% methanol (HPLC grade, Fisher scientific, USA) and further purified using high-performance liquid chromatography (HPLC). Chl *a* was used as a standard reference and was extracted from *Synechocystis* PCC 6803; Chl *d* was extracted from *A. marina* MBIC11017 as described in Schliep et al. [33]; Chl *f* was isolated from its containing cyanobacterium, a newly isolated cyanobacterium from red-light enrichment cultures [5]. Cells were harvested by centrifugation and rinsed in 40% methanol (v/v) to remove the extra retained water in the pelleted cells. The cell pellets were re-suspended in pre-chilled 100% methanol and kept on ice for 10 min, followed by 5 min centrifugation at top speed of a micro-centrifuge.

2.2. Purification of chlorophylls

The chlorophylls were pre-separated from other pigments in the raw pigment extraction on a Strata C18-T reverse phase column (200 mg/3 ml, Phenomenex, USA). The column was rinsed with 5 ml Milli-Q water and 5 ml 100% methanol, then equilibrated by 5 ml 80% methanol (v/v), before loading the raw pigment extract was equilibrated in 80% methanol. The loaded C18-T Column was washed

with 90% methanol until most of the yellow pigment was eluted, then the fractions containing chlorophylls were eluted in 100% methanol. All procedures were performed at 4 °C under normal gravity flow. The fractions containing chlorophylls were concentrated under a stream of N_2 gas, then directly subjected to HPLC analysis for further purification. All work was carried out rapidly and under dim green light illumination to minimize any possible photo-damage.

Final purification by HPLC was conducted on a Shimadzu VP series HPLC system with a reversed-phase C12 column (Synergi 4u MAX-RP 80A, 150 mm × 4.60 mm, 4 μ pore size, Phenomenex, USA). The column was equilibrated using 100% methanol and run with 100% methanol at 0.7 ml/min for 20 min. Eluted pigments were detected with a photodiode array detector (SPD-M10Avp, Shimadzu, Japan) at a wavelength range of 400 nm–800 nm. Purified chlorophylls were collected from HPLC peaks and the absorbance spectra of purified chlorophylls were immediately recorded using a Shimadzu UV-2550 spectrophotometer (Japan) at interval reading of 0.5 nm using a 1 cm square quartz cuvette covered by a lid.

2.3. In vitro absorbance spectral analysis of chlorophylls

Four different solvents were used in determining the spectrometric constants. 100% methanol (HPLC grade, Fisher scientific, USA), 100% acetone (ChromAR, Mallinckrodt chemicals, USA), 90% acetone (water:100% acetone (v/v) = 1:9), and 100% diethyl ether (AnalaR, Merck Pty. Limited, Australia).

Chlorophylls (Chl *d* and Chl *f*) were freshly prepared as described in Sections 2.1 and 2.2. Each of the HPLC purified chlorophylls was divided equally into 12 new glass vials. Methanol was removed by evaporation under a stream of N_2 gas and dried chlorophyll samples were re-dissolved in the new solvents to yield three replicates for each of the four testing solvents: 100% methanol, 100% acetone, 90% acetone and 100% diethyl ether, respectively. Chlorophylls with different solvent (~1 ml) were first transferred into the cuvette, then the volume was adjusted to the 1 ml mark labeled on the cuvette and covered by a lid immediately to avoid any evaporation during the measurement. Absorbance spectral analysis of Chl *d* and Chl *f* in different solvents was performed at room temperature and read in the range of 350–800 nm with a corrected baseline using the same solvent as blank. Each sample was read 3 times. Two sets of freshly prepared red-shifted chlorophyll samples were treated in this way. Thus each solvent yielded six replicates in total, two experimental samples each with three technical replicates. The spectral profiles presented are the mean of 18 spectral readings.

The purity of chlorophylls was re-checked after spectral analysis by HPLC system with the same setting as described above, but the flow rate of 100% methanol at 1 ml/min for 15 min.

2.4. ICP-MS measurement

All the glassware and plasticware used in the pigment extraction and purification described above were treated with 1 M HCl overnight, thoroughly rinsed with Milli-Q water and dried in an oven. Immediately after determination of the absorption maxima of the purified chlorophylls, each sample was dried under a stream of N_2 gas. Simultaneously, the same volume of solvent without pigment was also collected from HPLC and used as a blank for each sample. Sample vials were covered with aluminum foil to exclude light until digestion was commenced.

Chlorophyll *a* was freshly prepared as described in Sections 2.1 and 2.2 and divided into 10 new glass vials to generate a series of concentrations of Chl *a*, with extracted Mg concentration being in the range of 0.0–0.3 μg/ml. The absorption reading of Chl *a* at 665.5 nm was recorded using a Shimadzu UV-2550 spectrophotometer after zeroed at 750 nm and was immediately dried under a stream of N2 gas. Dried chlorophyll samples and the blanks were quantitatively digested by immersion of each in 1 ml of high purity 69% nitric acid (Merck) for one hour at room temperature. The samples and the blanks were then accurately spiked with 0.1 μg each of internal standard elements scandium, yttrium and indium, transferred and diluted with Milli-Q water to a final concentration of 5% (v/v) nitric acid. Prior to use, all disposable plastic items contacted with 69% nitric acid soaked for several hours in 10% (v/v) nitric acid then thoroughly rinsed with Milli-Q water.

Mg concentrations in the nitric acid extractions were measured with a Bruker (formerly Varian) 820-MS ICP-MS instrument (Kilsyth, Australia) fitted with a MicroMist concentric glass nebuliser and Peltier-cooled glass double-pass spray chamber. Plasma alignment, mass calibration and plasma optimisation were conducted daily. External calibration at *m/z* 24, 25 and 26 was carried out in the range of 0.1 to 100 ppb in normal sensitivity mode and without any detector attenuation. Internal standardisation was conducted using the instrument software's interpolation function.

3. Results

3.1. Separation of chlorophylls

The chlorophylls were pre-separated from other pigments, such as zeaxanthin and carotenes, by using a Strata C18-T reverse phase column (200 mg/3 ml, Phenomenex, USA). The column was washed by ~10 ml 90% methanol until most of the yellow pigment was eluted, and then the green fractions containing chlorophylls (diffuse green zone followed the yellow pigment) were separated and eluted by 100% methanol. For extractions containing Chl *f*, a diffuse faint green band near the bottom of the column after 90% methanol washing was mainly Chl *f*. This was eluted first in 100% methanol and was soon followed by the main dark green band that contains Chl *a*. The collected fractions were concentrated by N_2 streaming and purified chlorophylls were collected through HPLC at their retention time. The three chlorophylls were well separated by the HPLC isocratic elution with flow rate of 0.7 ml/min, Chl *a* has retention time of ~11.3 min, Chl *d* at ~7.2 min and Chl *f* at ~7.5 min. Due to the small amount of Chl *f*, several fractions were combined prior to HPLC purification. Chl *a* and Chl *d* fractions collected from Strata C18-T column can be up to 86–95% pure (data not shown).

3.2. Spectral properties in different solvents

The spectral profiles were obtained from the mean of 18 replicates and smoothed using the Savitzky–Golay method with window of 5 points using the Origin, version 8.0 software. Spectra were then re-plotted in Excel (Microsoft Office Excel 2007). The red-shifted chlorophylls, Chl *d* and Chl *f*, showed the expected red-shifted spectral profiles compared to Chl *a* in all four solvents (Fig. 2).

Chl *d* has three resolved absorption peaks at 400, 455.5 and 697 nm in 100% methanol, which agrees well with previous studies [4,34]. The two Soret bands have similar intensity and the ratio of Soret I:Soret II:Q_Y (I:II:Q_Y) is 0.7:0.7:1.0. Absorption in the solvents of 100% acetone and 90% acetone had a similar profile but with a 3 nm t red-shifted Q_Y of 691 nm in 90% acetone compared to the Q_Y of 688 nm in 100% acetone. The ratio of I:II:Q_Y is 0.6:0.9:1.0 in both 100% acetone and 90% acetone, and about 0.5:0.9:1.0 in 100% diethyl ether.

Chl *f* showed a diminished amplitude of the Soret II band around ~440 nm when it was dissolved in 100% methanol (Fig. 2B), which agrees well with the published spectrum of Chl *f* [5,35]. Chl *f* has three sharp absorption peaks at 395.5, 439.5 and 694.5 nm with the ratio of I:II:Q_Y of 0.7:0.7:1.0 in 100% diethyl ether. Spectral profiles observed for Chl *f* in 90% acetone and 100% acetone differed slightly in both relative intensity of the Soret bands and wavelength of maximum absorption of the Q_Y band. The Q_Y was red-shifted by 3 nm in

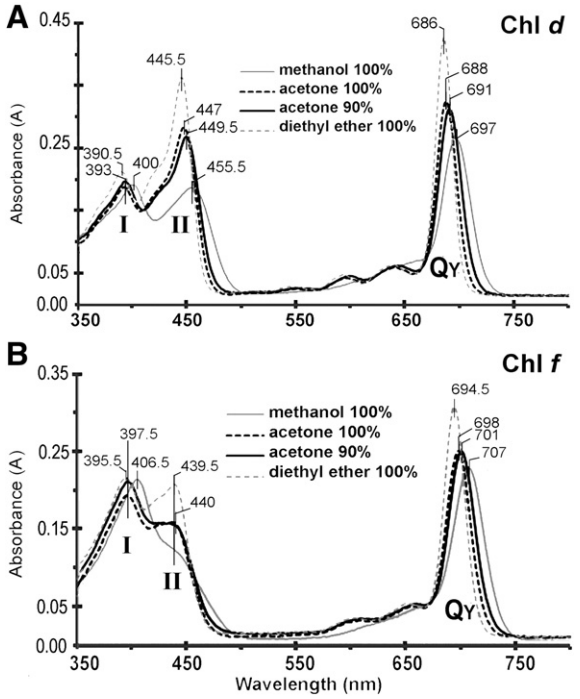


Fig. 2. Spectral comparison of purified chlorophyll *d* (Chl *d*) and chlorophyll *f* (Chl *f*) in different solvents. A, The absorption spectra of Chl *d* in 100% methanol, 100% acetone, 90% acetone and 100% diethyl ether; B, the absorption spectra of Chl *f* in 100% methanol, 100% acetone, 90% acetone and 100% diethyl ether. The wavelength maxima of absorption peaks were assigned after excel re-plotting. I, II and Q_Y represent the Soret bands I and II and the Q_Y transition band at red region. Solid black lines represent the spectra recorded in 90% acetone; Solid grey lines represent the spectra recorded in 100% methanol; black dashed lines represent the spectra recorded in 100% acetone; and grey dashed lines represent the spectra recorded in 100% diethyl ether.

90% acetone (λ_{QY} = 701 nm) compared with the Q_Y peak in 100% acetone (λ_{QY} = 698 nm).

For both Chl *d* and Chl *f*, the most red-shifted Q_Y *in vitro* wavelengths are in 100% methanol at 697 nm for Chl *d* and 707 nm for Chl *f*. Whereas 100% diethyl ether yields the most blue shifted Q_Y peaks, 688 nm for Chl *d* and 694.5 nm for Chl *f*. In addition the intensity of the Q_Y peak for both chlorophylls dissolved in 100% diethyl ether was about 1.4 times that of the Q_Y peak intensity in 100% methanol using the same concentration of purified chlorophylls (Fig. 2B).

3.3. ICP-MS measurement

A series of concentrations of Chl *a*, with extracted Mg concentration being in the range of 0.0–0.3 μg/ml, were prepared for a standard curve representing the referred relationship between the absorption reading of Chl *a* at 665.5 nm (Q_Y maximum) and Mg concentration determined by ICP-MS. As all Mg isotopes of *m/z* 24, 25 and 26 gave nearly identical values, the results for the most abundant isotope *m/z* 24 were used here. The actual Mg concentration was obtained by normalizing to subtract the reading generated from blank samples. Fig. 3 represents a standard curve obtained using purified Chl *a* and its acid extraction Mg concentration determined by ICP-MS. A linear relationship between Mg concentration determined by ICP-MS and the absorbance reading ($A_{665.5\ nm}$) of Chl *a* is presented. Therefore, we aim to measure the Mg concentration from Chl *d* and Chl *f* in the range of 0.1–0.3 μg/ml.

Table 1 lists the absorption readings of different chlorophylls at the indicated wavelength. All readings were a single measurement based on the replicates (n) and were obtained after zeroing at 750 nm. All Mg concentrations reported in Table 1 were determined after subtracting the Mg concentration of the blank samples. Each result is the mean of replicates, which is presented as 'n' in the

Mg (C,μg/ml) measured by ICP-MS	Chl *a* (A) at 665.5nm
0.04058	0.1244
0.13273	0.3772
0.17399	0.4975
0.21802	0.6219
0.26218	0.7463
0.26052	0.7463

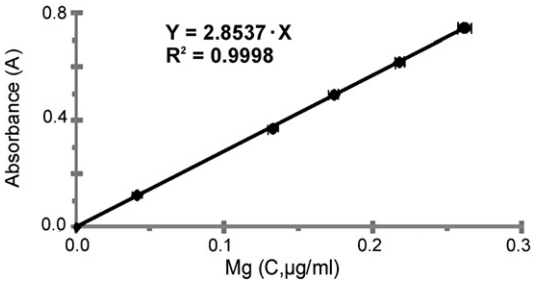


Fig. 3. Standard curve represents the relationship between the Mg concentration in Chl *a* determined by ICP-MS and its absorbance maxima (Q_Y) (λ = 665.5 nm). The error bars presented here are estimates of the total method imprecision and are the same as the RSD presented in Table 1.

brackets. The relative standard deviation (RSD, %) of the mean (m) is presented as $100 \times \sigma/m$, where σ represents the standard deviation which was calculated by Excel (Microsoft office Excel 2007) using the formula STDEV. All data were verified by the $<\pm 1.4\%$ RSD percentage of mean (Table 1).

3.4. Determination of extinction coefficients

The molar extinction coefficient (ε) can be determined using equation: $\varepsilon = A/c \cdot l$, which was converted based on the Beer–Lambert law, $A = \varepsilon \cdot c \cdot l$, where A represents an actual absorption at the given wavelength, path length *l* is 1 cm in our study, and the molar concentration *c* was obtained from Mg concentration (C_{Mg}, μg/ml) determined by ICP-MS. *i.e.* $\varepsilon_{\lambda nm} = A_{\lambda nm} \cdot 24.30/C_{Mg}$ (μg/ml) × 1000, where 24.30 was used as the mass of Mg atom. The mass extinction coefficient (α) was calculated based on $\alpha = \varepsilon$/chlorophyll molecular mass, where 893.5, 895.5 and 907.5 were used as the molecular mass of Chl *a*, Chl *d* and Chl *f*, respectively.

Table 2 presents the extinction coefficients of Chl *a*, Chl *d* and Chl *f* using 100% methanol as dissolving solvent. All the absorption readings at different wavelengths were referenced by zero at 750 nm, which is the same as described in Porra et al. [22]. The mass extinction coefficients (α, L g^{-1} cm^{-1}) were calculated based on the molar extinction coefficient (ε, L mol^{-1} cm^{-1}). The absorbance coefficients (E_λ, mg L^{-1} cm) are $1/\alpha$, where λ is the wavelength (nm). Therefore, the accuracy of α should be identical to ε, *i.e.* they have the same RSD because they were derived from the same set of data presented in

Table 1
Mg concentration of Chl *a*, Chl *d* and Chl *f* and their absorption at the absorption peaks of chlorophylls.

Chlorophylls	Mg concentration measured by ICP-MS (C, μg/ml)	Wavelength (nm)	Absorption readings (A) (n = replicates, ± RSD %)
Chl *f*	0.2403 (n = 10, ± 1.26)	707.0	0.7030 (n = 4, ±`0.32)
		406.5	0.6616 (n = 4, ± 0.79)
Chl *d*	0.1403 (n = 10, ± 0.95)	697.0	0.3677 (n = 4, ± 0.36)
		455.5	0.2564 (n = 4, ± 0.20)
		400.0	0.2641 (n = 4, ± 0.15)
Chl *a*	0.04317 (n = 6, ± 1.30)	665.5	0.1244 (n = 5, ± 1.35)

Errors are the relative standard deviation (RSD, %) of the mean (m), $100 \times \sigma/m$, where σ represents the STDEV calculated by Excel.

Table 2
Extinction coefficients of chlorophylls in 100% methanol.

Chlorophylls	λ_{max} (nm)	ε (L mol^{-1} cm^{-1})	α (L g^{-1} cm^{-1})	E_λ (mg L^{-1} cm)[f]
Chl *f*	707.0[a]	71.11×10^3	78.36	12.76
	406.5[a]	66.92×10^3	73.74	13.56
Chl *d*	697.0[a]	63.68×10^3	71.11	14.06
	696.0[b]	–	77.62 ± 0.36	12.88
	455.5[a]	44.41×10^3	49.59	20.17
	400.0[a]	45.74×10^3	51.08	19.58
Chl *a*	665.5[a]	70.02×10^3	78.36	12.76
	666[c]	–	79.29	12.61
	665.2[d]	70.54×10^3	–	12.67
	665.2[e]	$71.43\times10^3\pm0.91$	79.95	12.51

[a] Current study.
[b] Ritchie [23].
[c] Lichtenthaler and Wellburn [36].
[d] Lichtenthaler [37].
[e] Porra et al. [22].
[f] E_λ is calculated based on E_λ (mg L^{-1} cm) = 1000/α (L g^{-1} cm^{-1}) and α (L g^{-1} cm^{-1}) = ε (L mol^{-1} cm^{-1})/chlorophyll molecular weight.

Table 1. The extinction coefficient of Chl *a* agrees well with all the published result [22,36,37], with less than 2% variation (Table 2).

3.4.1. The extinction coefficients for Chl d and Chl f in selected solvents

According to the Beer–Lambert law, $A = \varepsilon \cdot c \cdot l$, when '*c*' and '*l*' are same among different sample, $A_1/A_2 = \varepsilon_1/\varepsilon_2$. According to ε for Chl *d* and Chl *f* in 100% methanol at certain wavelengths (Table 2), ε for Chl *d* and Chl *f* in other solvent were calculated based on the ratio between the absorbance at different maximum absorption wavelengths (A_λ) and are presented in Table 3. As described above, all A_λ were referenced by zeroing at 750 nm. Results are the mean of 18 replicate samples (n = 18). The RSD percentages were less than ±1%, which proves the reliability of ε and α value in the table (Table 3).

For Chl *d*, the mass extinction coefficient in 100% diethyl ether is 116.64 (L g^{-1} A_{686nm} cm^{-1}) (n = 18, ±0.56). Compared with the data reported in Smith and Benitez [21], which presents as 110.4 (L g^{-1} A_{688nm} cm^{-1}), this value in our result is ~5.7% higher, also, the λ_{max} is ~2 nm blue shifted. According to the absorption curve of Chl *d* in 100% diethyl ether presented in 1943 by Manning and Strain [4], the λ_{max} of Q_Y was also presented as 686 nm, which agrees with the result in this paper. We are presenting the first report for extinction coefficients of Chl *f* based on ICP-MS data.

4. Discussion

Evaporation of solvents during transfer and measurement is potentially one of the largest sources of error in determination of extinction coefficients, especially for low boiling point solvents like diethyl ether. This error was minimized by adjusting the volume to 1 ml after transferring the sample into measuring cuvette followed by rapidly sealing with a lid secured in place with parafilm.

To minimize the oxidation or degradation of chlorophylls, all procedures were carried out under dim green light to minimize light-associated damage of chlorophylls and keep them at 4 °C. All samples were freshly prepared and dried by a stream of N_2 and stored at −80 °C. The purity of isolated chlorophylls after spectral measurement was confirmed by re-checking using HPLC analysis with a flow rate of 100% methanol at 1.0 ml/min (Fig. 4). The dried chlorophylls and the chlorophylls after solvent replacement were re-loaded to HPLC and showed that variable micro contaminations, such as other pigments or oxidation products of chlorophylls, were less than 2% of purified chlorophylls (arrows in Fig. 4). The higher purity of chlorophyll *d* in this study may contribute to the slightly higher $\alpha_{686\ nm}$ of 116.64 (L g^{-1} cm^{-1}) than 110.4 (L g^{-1} cm^{-1}) in 100% diethyl ether reported by Smith and Benitez in 1955 [21]. Pigment contaminations such as oxidation or degradation products of chlorophylls were inseparable, but could contribute to the absorption reading. The derived spectrometric equations based on a previous report and the Chl *d* molecular weight of 894.5 (895.5 for Chl *d* was used in this study) estimated a lower coefficient E_{696nm} = 12.9367 ± 0.36% μg ml^{-1} cm in 100% methanol [23]. According to this study, the E_{697nm} should be 14.06 ± 0.36% μg ml^{-1} cm in 100% methanol (Table 2).

The application of ICP-MS provides an advantage in this study, quantitation of Mg from very small amounts of purified chlorophylls. For Chl *a* and Chl *d*, which are the major chlorophylls in *Synechocystis* PCC 6803 [1] and *A. marina* MBIC11017 [6], there is no difficulty for extraction and purification of large quantities chlorophylls. However, for Chl *f* which comprises just about 10% of total chlorophylls in its containing cyanobacterium [5], the optimum culture condition for the Chl *f*-containing organism is still under testing. Fortunately, ICP-MS could provide us with quantitation of Mg down to 1 ppb, which met the quantity of Chl *f* that we could prepare. Meanwhile, this helped us eliminate the need for large scale extraction and purification of chlorophylls (approximately 15 mg of Chl *d* prepared in Smith and Benitez 1955), which is a complicated and lengthy

Table 3
Extinction coefficients of Chl *d* and Chl *f* in selective solvents.

Chlorophyll	Solvent	λ_{max} (nm)	ε (L mol^{-1} cm^{-1})	α (L g^{-1} cm^{-1})
Chlorophyll *d*	100% acetone	688.0	78.77×10^3 (n = 18, ±0.62)	87.97
		447.0	68.16×10^3 (n = 18, ±0.43)	76.11
		393.0	43.89×10^3 (n = 18, ±0.46)	49.01
	90% acetone	691.0	75.43×10^3 (n = 18, ±0.19)	84.23
		449.5	64.26×10^3 (n = 18, ±0.08)	71.76
		393.0	46.13×10^3 (n = 18, ±0.11)	51.51
	100% diethyl ether	686.0	104.5×10^3 (n = 18, ±0.56)	116.64
		445.5	88.38×10^3 (n = 18, ±0.36)	98.69
		390.5	50.06×10^3 (n = 18, ±0.39)	55.90
		688.0[a]	–	110.4
		686.0[b]	–	–
Chlorophyll *f*	100% acetone	698.0	77.97×10^3 (n = 18, ±0.16)	85.91
		439.5	48.04×10^3 (n = 18, ±0.25)	52.94
		397.0	59.31×10^3 (n = 18, ±0.46)	65.35
	90% acetone	701.0	77.89×10^3 (n = 18, ±0.05)	85.83
		439.5	46.97×10^3 (n = 18, ±0.14)	51.76
		397.5	65.08×10^3 (n = 18, ±0.27)	71.71
	100% diethyl ether	694.5	96.62×10^3 (n = 6, ±0.45)	106.47
		439.5	64.09×10^3 (n = 6, ±0.28)	70.63
		395.5	66.96×10^3 (n = 6, ±0.40)	73.78

The errors presented here are the RSD (%) of the mean (m) as 100 × σ/m, where σ represents STDEV calculated by Excel (Microsoft office Excel 2007).
[a] Smith and Benitez [21].
[b] Manning and Strain [4].

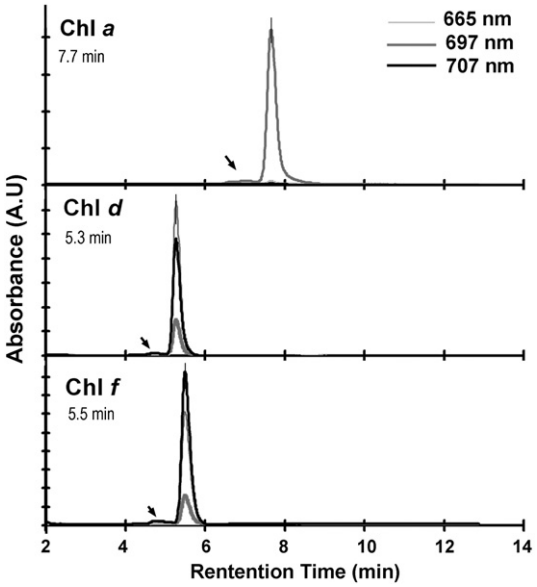


Fig. 4. HPLC chromatography of Chl *a*, Chl *d* and Chl *f* samples after experiments. The chlorophyll samples, which were in different solvents for absorption reading, were dried by N_2 gas streaming and dissolved in 100% methanol, then directly subjected to HPLC analysis at flow rate of 1.0 ml/min. The retention time of each chlorophylls showed different retention times: Chl *a*, 7.7 min; Chl *d*, 5.3 min and Chl *f*, 5.5 min. Black line represents the chromatography detected at 707 nm; thick grey line represents the chromatography detected at 697 nm; and light grey line represents the chromatography detected at 665 nm. Arrows indicated the oxidative products formed during the experimental procedure, which is less than 2% of main chlorophylls.

procedure, requiring a lot of resources (*e.g.* solvents). According to the result of this paper, our method provides an efficient and shorter route with the same or better accuracy.

As the molar concentration of Mg represents the molar concentration of chlorophylls, the amount of Mg in Chl *a* determined by ICP-MS shows an identical linear relationship with the gradient increased concentration of chlorophylls with R = 0.9998. The extinction coefficients for Chl *a* in 100% methanol at its absorption maxima of 665.5 nm were verified in this paper using ICP-MS, with a variation of less than 2% compared with published data [22,36,37]. The methodology developed here will be also useful for determining extinction coefficients of various bacteriochlorophylls containing Mg. In our experiment, this advanced atomic spectroscopy technique was first applied in qualitative determination of chlorophylls, and the efficiency of Mg determination in chlorophylls using ICP-MS has been verified.

5. Conclusion

The extinction coefficient is known to be an important parameter that determines the photosynthetic reactions based on the involvement of chlorophylls. In this study, extinction coefficients of Chl *d* and Chl *f* in four selected solvents were determined for the first time by combining spectral analysis and ICP-MS methods. The extinction coefficient of Chl *f* in 100% methanol is $\varepsilon_{707\ nm} = 71.11 \times 10^3 \pm 0.32\%\ L\ mol^{-1}\ cm^{-1}$ and $\alpha_{707\ nm} = 78.36 \pm 0.32\%\ L\ g^{-1}\ cm^{-1}$; The extinction coefficient of Chl *d* in 100% methanol is $\varepsilon_{697\ nm} = 63.68 \times 10^3 \pm 0.36\%\ L\ mol^{-1}\ cm^{-1}$ and $\alpha_{697\ nm} = 71.11 \pm 0.36\%\ L\ g^{-1}\ cm^{-1}$. Based on this study, the extinction coefficient of Chl *a* in 100% methanol is corrected as $\varepsilon_{665.5\ nm} = 70.02 \times 10^3 \pm 1.35\%\ L\ mol^{-1}\ cm^{-1}$. 100% diethyl ether showed the sharpest dipole intensity for Chl *d* and Chl *f*. 100% methanol demonstrated the most red-shifted Q_Y transition, 697 nm for Chl *d* and 707 nm for Chl *f*.

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